

Radiological, Morphological, Histological and Biochemical Changes of Lumbar Discs in an Animal Model of Disc Degeneration Suitable for Evaluating the Potential Regenerative Capacity of Novel Biological Agents

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Abstract

Circumferential tears of the Annulus Fibrosus (AF) are frequently observed pathological features of degenerate lumbar discs and have been associated with vascular propagation and the generation of low back pain. In order to evaluate the potential of novel biological agents to repair annular defects and arrest disc degeneration we required an animal model that would permit injection of cells or their cryoprotectant into adjacent lumbar discs of the same animal. Three lumbar discs (L2/3, L3/4 and L4/5) of 6 sheep were subjected to a peripheral lateral annular surgical incision. The adjacent uninjured lumbar L1/2 and L5/6 discs served as uninjured controls. After three months the spines were radiographed and disc height indices (DHI) calculated and Pfirrmann disc degeneration scores determined from MRI spinal images. Isolated lumbar discs were analysed morphologically, histologically and biochemically using published procedures.

Disc height index measurements of injured discs revealed an average decrease of 23.67% relative to baseline values ($p < 0.0001$). The corresponding MRI Pfirrmann degeneration scores were significantly higher than non-injured control discs ($p < 0.05$), as were their morphology scores ($p < 0.005$). The sulphated - glycosaminoglycan content, of the Nucleus Pulposus (NP) and injured side of the AF of lumbar discs, were significantly lower than control discs ($p < 0.05$ and $p < 0.0005$) respectively. Conversely, the DNA levels of the injured side of the AF were higher than the uninjured side ($p < 0.05$). The histological scores showed higher degenerative changes in injured than in control discs ($p < 0.005$).

For all parameters monitored in this study no statistical differences were observed between the three injured lumbar discs confirming their uniform response to injury. This study therefore confirmed the suitability of this large animal model for evaluating the potential of biologicals to reconstitute degenerate ovine lumbar discs relative to their carriers/cryoprotectant.

Keywords: Peripheral lesions of the annulus fibrosus; Multilevel lumbar disc degeneration; Potential biological agents; Radiological; Morphological, histological and biochemical studies of lumbar discs

Introduction

Low back pain is the most common cause of disability worldwide and represents an enormous socioeconomic problem and financial burden on national healthcare providers [1]. The intervertebral disc has been implicated in the generation of low back pain directly, (discogenic pain), arising from the pathological innervation of the inner AF [2,3]. Degenerate discs may also promote pain indirectly through their failure to act efficiently as a functional spinal unit (FSU). As a consequence adjacent structures, such as the facet joints, are overloaded and become osteoarthritic [4-6]. Furthermore, loss of disc height arising from annular protrusion or prolapse can impinge upon nerve roots within the foraminal space resulting in local inflammation and the generation of radicular pain [7,8].

There are a multitude of conservative methods for managing low back pain including, analgesics, non-steroidal anti-inflammatory drugs, physical therapies and enrolment in pain management programs. Patients with chronic low back pain who fail to respond to conservative treatments generally resort to surgical interventions, such as laminectomy, lumbar fusion or total disc arthroplasty. However,

while these procedures can provide relief of symptoms, some patients still experience back pain, particularly at adjacent untreated spinal levels [8].

Recent advances in tissue engineering and stem cell research has stimulated investigators, to examine biological strategies that might delay the degenerative process occurring within the disc, or to even abrogate or reverse their progression. Preclinical studies to evaluate the efficacy and safety of these novel regenerative therapies requires the use of animal models of disc degeneration that ideally would simulate, the known pathobiology of human disc degeneration.

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The Intervertebral Disc (IVD) is a composite tissue located between the adjacent vertebrae of the spinal column. Healthy discs consist of an outer fibro-cartilaginous structure, the Annulus Fibrosus (AF) that encapsulates a central hydrated gelatinous centre, the Nucleus Pulposus (NP). The interface between the disc and the vertebral bodies is occupied by the hyaline Cartilage End Plates (CEP) through which pass the fibrous attachments of the AF. The majority of the AF is composed of concentric multilayers cylinders (lamellae) in which the tendon-like collagen fibres are aligned obliquely crossing from one vertebral body to the next. Significantly, the main collagen fibre orientation within each adjacent lamellae cross at an angle of approximately 90°, an arrangement that lowers the distribution of mechanical stresses on axial rotation and loading of the disc [9,10].

The biomechanical function of the IVD is to facilitate movement and flexibility of the vertebral column, whilst also maintaining its ability to recover from deformation following axial loading. Excluding water, the predominant extracellular matrix components of the disc are proteoglycans and collagens. The collagen of the NP is predominantly type II collagen, whilst the AF is composed of both type I and type II collagens. Topographically the inner AF contains mainly type II collagen, while the outer AF is largely type I [9–13]. The NP contains high quantities of proteoglycans, which, because of their macromolecular size and high anionic charge, retain large amounts of water within the NP [9,11–14]. This imbibition of water is critical for the normal biomechanical functions of the disc, allowing it to resist compression, recover from deformation and transmit axial loading to the annulus [9,13–15].

The causes of disc degeneration are multifactorial and include aging (cell senescence), hormonal, genetic, nutritional and mechanical factors. An imbalance between the rate of degradation of components of the extracellular matrix and their biosynthesis results in progressive structural failure of the disc accompanied by altered biomechanical function at the affected spinal level [8,11–17]. Furthermore, alterations in disc cell number and viability, and adverse cellular responses to nutritional deficiencies, can in turn, and lead to a disturbance in the normal metabolism of components of the extracellular of the disc. Apart from a decline in matrix synthesis due to apoptosis and reduced viability of disc cells [17], cytokines released by activated NP cells up-regulate the production of proteinases, including the aggrecanases and Matrix Metallo-Proteinases (MMP-3, MMP-8, MMP-13) that possess the capacity to degrade the **Proteo Glycans** (PGs) and collagens of the disc [11,13,18–21]. Turnover of the collagenous components of the disc also occurs with aging and degeneration where the levels of type II collagen decline, accompanied by deposition with collagen types I, III, VI and X [11,12,22].

Capillaries located at the periphery of the annulus and penetrating the CEP beneath the NP provide the major routes for exchange of nutrients and metabolites with cells of the disc and the blood supply. However, as the disc is the largest avascular structure in mammals the majority of NP cells reside in a precarious nutritional environment, relying on the efficient molecular diffusion of solutes through the CEP from the vertebral body for their survival [11,14,23].

Once disc degeneration becomes established the capillary plexus of the outer AF may proliferate penetrating the inner disc regions and eventually to the NP [2,24,25]. This induced ingrowth of blood vessels and nerve fibres from the outer AF into the avascular regions of the disc has been cited as contributing to the cause of back pain [2,24,25]. However, with aging and degeneration structural changes may also occur in the CEP, such as thinning and calcification, resulting in the

deterioration in the normal exchange of solutes between the NP and the vasculature within the vertebral body [11,14,23]. The situation may be exacerbated by degenerative changes in the adjacent vertebral bodies including trabecular micro-fractures, sclerosis and bone marrow oedema [8,26].

Ideally, an animal model of disc degeneration would mimic the human degenerative process in terms of the cellular, matrix and biomechanical changes described above. Given the complex nature of human disc degeneration, its multifactorial aetiologies and lengthy time-course, an animal model that parallels the human condition is not feasible. Nonetheless, many animal models using a variety of species have been described and used to investigate the underlying pathobiology of disc degeneration and to evaluate potential therapeutic modalities to facilitate its repair [27–31].

Despite being a quadruped, the sheep spine exhibits many anatomical, physiological, biochemical and biomechanical similarities to the human spine rendering it increasingly popular as a large animal model for preclinical spinal surgery studies [32–34]. Importantly, with aging sheep lumbar discs, like those of the chondrodystrophoid dog and the human disc, undergo a decline in the number of notochordal cells remaining in their NP; a genetically determined developmental process that predisposing discs to early degeneration and dystrophic calcification [35,36].

The ovine model of disc degeneration induced by annular injury/incision was first described in 1990 [37]. In this model the temporal effects of a limited controlled horizontal incision (5 mm x 5 mm) initiated in the periphery of the annulus that extended laterally, but did not penetrate the NP, was investigated over a period of 4–18 months [38]. These studies demonstrated relatively good healing potential of the very outer third of the annular lesion, but the lesion was observed to propagate into the avascular regions of the disc as radiating and concentric clefts, eventually leading to degeneration of the entire disc [37,38].

Pathological studies on human specimens have shown that similar types of annular rim lesions to those observed in the sheep model occur in degenerate discs [38], the frequency of which increases with aging [39,40]. Whether these lesions are the primary cause of human disc degeneration, or are secondary to earlier degenerative changes in the NP and/or CEP, remains unresolved. Irrespective of the order, it is clear that any violation of the integrity of the AF would disturb the biomechanics of the disc, thereby promoting cellular responses and matrix remodelling.

More recently Melrose et al. [41] reported that induction of a 20 mm by 6 mm horizontal annular incision in the sheep disc at a single lumbar level promoted degenerative changes within a three-month period without spontaneous enucleation of the NP [41]. The induction of significant disc degenerative changes within three months rendered this modification attractive since it would facilitate the evaluation *in vivo* of novel regenerative biological agents in a shorter timeframe compared to the original Osti et al model [37].

In the present study, we utilised the Melrose modified surgically induced annular injury lesion model [41] to determine the radiological, morphological, histological and biochemical outcomes at three adjacent levels of the lumbar ovine spines in order to use it as for the preclinical evaluation of Mesenchymal Precursor Cells (MPC) relative to their cryoprotectant vehicle in spines of the same animals.

Materials and Methods

Study design

Six adult East Friesian/Merino male sheep were subjected to a

standardized 6 mm x 20 mm annular incision at 3 consecutive lumbar levels – L2/3, L3/4 and L4/5 – (n=18). The adjacent L1/2 and L5/6 lumbar discs (n=12) served as uninjured controls. Necropsies were undertaken at 12 weeks following injury and the spines isolated *en-bloc*. All spines underwent radiological analysis using x-ray and MRI, and then cut horizontally through adjacent vertebral bodies into spinal segments using a band saw. Intervertebral disc segments from three spines were subjected to morphological and biochemical analysis, and three for histological studies.

Experimental surgery

All surgical and experimental procedures used in this study were approved by the Monash Medical Centre Animal Ethics Committee and conforming to the Australian code of practice for the care and use of animals for scientific purposes 7th Edition, 2004.

Sheep were fasted for 24 hours and anaesthetised using intravenous thiopentone (10-15 mg/kg) and isoflurane inhalation (2-3% in oxygen) prior to being placed in the right lateral position. Following the subcutaneous administration of local anaesthetic (bupivacaine 0.5%), the L2/3, L3/4 and L4/5 lumbar intervertebral discs were exposed using a minimally invasive left lateral retroperitoneal approach described previously [42]. Intraoperative lateral x-rays were performed to identify the correct levels and also obtain digital images for calculation of Disc Height Indices (DHI).

The L2/3, L3/4 and L4/5 discs were all subjected to a standardised annular incision procedure [42]. Using a custom scalpel, with 6 mm depth collar attached, a horizontal 6 mm x 20 mm annular incision was made in the left anterolateral aspect of the L2/3, L3/4 and L4/5 intervertebral discs. A routine layered closure was then performed using absorbable sutures (Vicryl™, Ethicon USA). Animals received a fentanyl patch (Durogesic® 75mcg/hr) for post-operative analgesia and once recovered were immediately returned to deep litter holding pens with other sheep. After one week and following veterinarian approval, animals were transferred to open pasture for three months.

Twelve weeks following surgery animals were humanely euthanased by a clinical veterinarian using an intravenous injection of 150 mg/kg of pentobarbitone. The lumbar spines were removed *en bloc*, by transecting the spine from the sacral midpoint to the thoracolumbar junction, for subsequent radiological, biochemical and histological analysis.

Radiographic analysis

Lateral lumbar spinal digital x-rays (Radlink, Atomscope HF 200A, Redondo Beach CA) of all sheep were obtained pre-operatively and prior to post-mortem assessment with the lumbar spine *in situ*. Using a standardized method [31], Disc Height Index measurements were calculated by a Blinded Observer (DO) using digital image processing software (Osirix X v4.1.2).

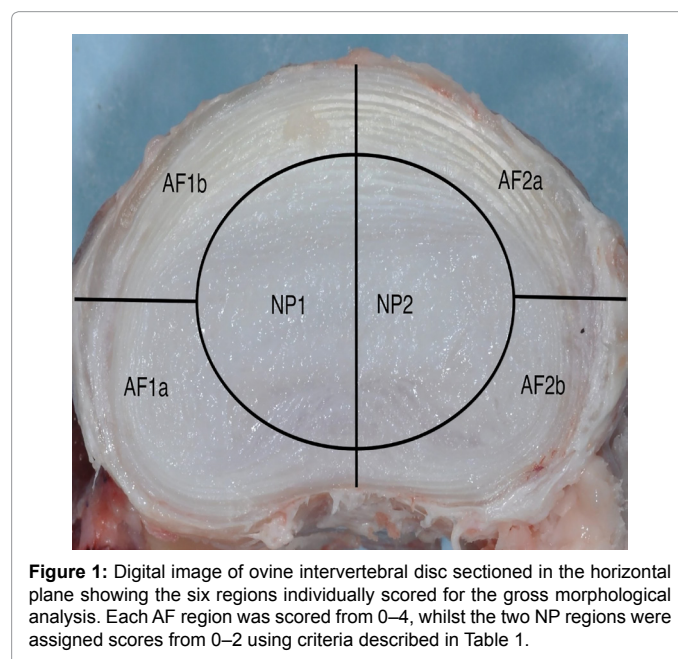
Immediately following necropsy the lumbar spines were harvested from the thoracolumbar junction to the midpoint of the sacrum, and transferred to Monash Biomedical Imaging (MBI, Melbourne, Australia) for MRI analysis (Siemens 3-T Skyra Widebore MRI). 3T sagittal and axial T2-weighted MRI sequences of the entire lumbar spine explant (L1-S1) were acquired for each animal. Using sagittal T2 weighted sequences, three blinded observers (radiologist – SS, neurosurgeon – TG, neurosurgery resident – DO) determined the Pfirrmann and modified Pfirrmann MRI disc degeneration scores for all lumbar discs [43,44].

Disc morphology assessment

Discs from spinal groups destined for biochemical analysis were used for gross morphological assessment. Following routine sectioning of the disc in the horizontal (axial) plane, high-resolution digital photographs were taken of the exposed complementary surfaces and each scored by a blinded observer utilizing a newly developed disc morphological grading system. In this grading system, the Annulus Fibrosus (AF) was divided into four quadrants (Figure 1), and each quadrant scored separately from 0–4, according to criteria outlined in Table 1. AF1a and AF1b regions were on the injured (left) side of the disc, whilst AF2a and AF2b were contralateral to the injury. The Nucleus Pulposus (NP) was divided in two and each half (NP1 and NP2) scored separately from 0–2, according to criteria outlined in Table 1. The sum of all component scores yielded a total disc degeneration score from 0 (no degeneration) to 20 (severe degeneration).

Biochemical analysis

Following dissection of the lumbar disc segments and collection of the digital images from each spinal level for morphological analysis, the tissues were subjected to biochemical analysis. The Nucleus Pulposus (NP) and annular (AF) regions were identified by their different morphological appearances and were separated from each other and their vertebral attachments by careful dissection. The AF tissues were subdivided into two halves in the sagittal plane, AF1 (annular half containing the annular injury) and AF2 (annular half contralateral to the annular injury). Tissues were then finely diced, weighed, lyophilised and reweighed to determine their dehydrated mass. All the dehydrated NP, AF1 and AF2 tissues were subsequently solubilised using a papain digestion buffer (50 mM sodium acetate, 25 mM EDTA and 10 mM Cysteine containing 2 mg/mL papain - Sigma-Aldrich Chemical, Sydney, Australia, at pH=6.0) at 60°C for 16 hours [45]. The digested tissues were then centrifuged at 3000 g for 15 minutes and supernatants diluted to a standard volume (the stock digest solution). Aliquots of the stock solution were analysed for Sulphated Glycosaminoglycans (S-GAG) (an index of proteoglycan content) [46], Hydroxyl Prolene (HP) (to calculate collagen content) [47] and DNA content [48] as an index of cell numbers.



AF Quadrant Scoring System		NP Scoring System	
0	Normal Disc: Concentric lamellated AF structure maintained without disruption or discoloration. No distortion between AF/NP transition zone.	0	Normal NP: No discoloration or haemorrhage. Gelatinous hydrated appearance.
1	Minor Disruption: Minor disruption/discoloration of lamella in outer half of AF zone. Preservation of inner half of AF zone and AF/NP transition zone.	1	Minor Disruption: Discoloration and/or haemorrhage < 50% NP region. No fissuring. Some evidence of nuclear dehydration.
2	Moderate Disruption: Moderate disruption /discoloration of outer half of AF extending into inner half. Preservation of AF/NP transition zone.	2	Major Disruption: Discoloration and haemorrhage > 50% NP region, and/or presence of fissuring. Dehydrated appearance.
3	Major Disruption: Disruption/discoloration of entire AF with loss of AF/NP transition zone.		
4	Complete Disruption: Complete loss of lamellated structure and AF/NP transition zone with extension into NP. Bulging or prolapse of degenerate disc tissue.		

Table 1: Disc morphology criteria used to score each NP and AF quadrants shown in [Figure 3](#).

Histological analysis

Individual disc segments were prepared from the spinal columns of each sheep using a bandsaw. After removing the spinous processes and spinal canal adjacent vertebral bodies were all bisected in the horizontal plane. The intact lumbar disc, with caudal and cranial sections of the vertebral bodies attached were fixed in multiple changes of 10% neutral buffered formalin for 8 days, prior to transfer to 70% ethanol. The fixed disc segments were then transported to Ratliff Histology Consultants (Franklin, TN, USA) for methylmethacrylate resin-based tissue embedding. Coronal sections of entire disc segments were cut using a motorized sliding microtome and stained using Haematoxylin and Eosin (H and E), Goldner's Trichrome (GT) and Safranin-O/Fast Green (SO), using standard procedures. Stained sections were automatically scanned using an Aperio Scanscope AT-Turbo system and the stored digital images then examined using Aperio Imagescope-Rev-v12 software (Aperio Technologies, Vista, California, USA). Selected images were downloaded as tiff files.

A semi-quantitative ovine lumbar intervertebral disc histological grading system, based on a published canine histological grading system [28] was used to assess the degenerative changes in the collected tiff files of the disc sections. Sections were scored separately by a blinded board certified veterinary pathologist (MW) by examination of sections from the two complementary halves of each disc (e.g., AF1 and AF2). Scores incorporated all the any disc elements (AF, NP, cartilaginous endplates (CEP) and adjacent vertebral bodies (VB) as listed in Table 2. The sum of all component scores (AF, NP, CEP, VB) from both halves of the disc yielded a total disc histological score ranging from 0 (no degeneration) to 54 (extreme degeneration).

Statistical analysis of Data

All data processing and statistical analysis was performed using Excel (2011, Microsoft Corporation) software and Prism 5.0d (Graph Pad Software). Parametric data were analysed using one-way analysis of variance (ANOVA) and Tukey's Multiple Comparison Test was performed when significant differences in means were observed. Non-parametric data were analysed using Kruskal-Wallis test of median values followed by Dunns Multiple Comparison Test. Injury and control groups were compared using the two-tailed student t-test followed by Mann Whitney analysis. A p-value of <0.05 was considered statistically significant.

Results

Disc height analysis

Discs subjected to annular incision 12 weeks previously showed a significant decrease of DHI of 23.67% relative to the baseline values

($p < 0.0001$) (Figure 2A). Notwithstanding differences in mean values, there were no statistical differences between the two control discs (L1/2 and L5/6), nor between any of the three discs subjected to annular incision (L2/3, L3/4, L4/5) (Figure 2B) confirming the suitability of this model for evaluating the efficacy of biological therapies relative to their vehicles in the same animal.

MRI analysis

Median Pfirrmann MRI degeneration scores were significantly higher in injured discs compared to non-injured controls ($p < 0.05$) (Figure 3A). There were no statistical differences in Pfirrmann scores between the three levels subjected to annular incision (L2/3, L3/4, L4/5) (Figure 3B) as was observed for the DHI evaluations. An example of the MRI changes observed for the injured and control discs in this study is shown in Figure 4.

Disc morphology

Morphology scores were significantly higher in injured discs, compared to non-injured controls ($p = 0.048$) (Figure 5A). There were no statistical differences in scores between control discs (L1/2 and L5/6), or between the three levels undergoing annular incision (L2/3, L3/4, L4/5) (Figure 5B).

Biochemical analysis

The S-GAG content, determined as percentage dry weight, of the NP regions of all injured discs was significantly less than the amounts present in the NP of uninjured control discs ($p < 0.05$) (Figure 6A) a result consistent with the DHI and MRI findings and indicative of a decline in proteoglycan and water content in these tissues. No significant differences were observed in S-GAG content of NPs from the injured discs (Figure 6B).

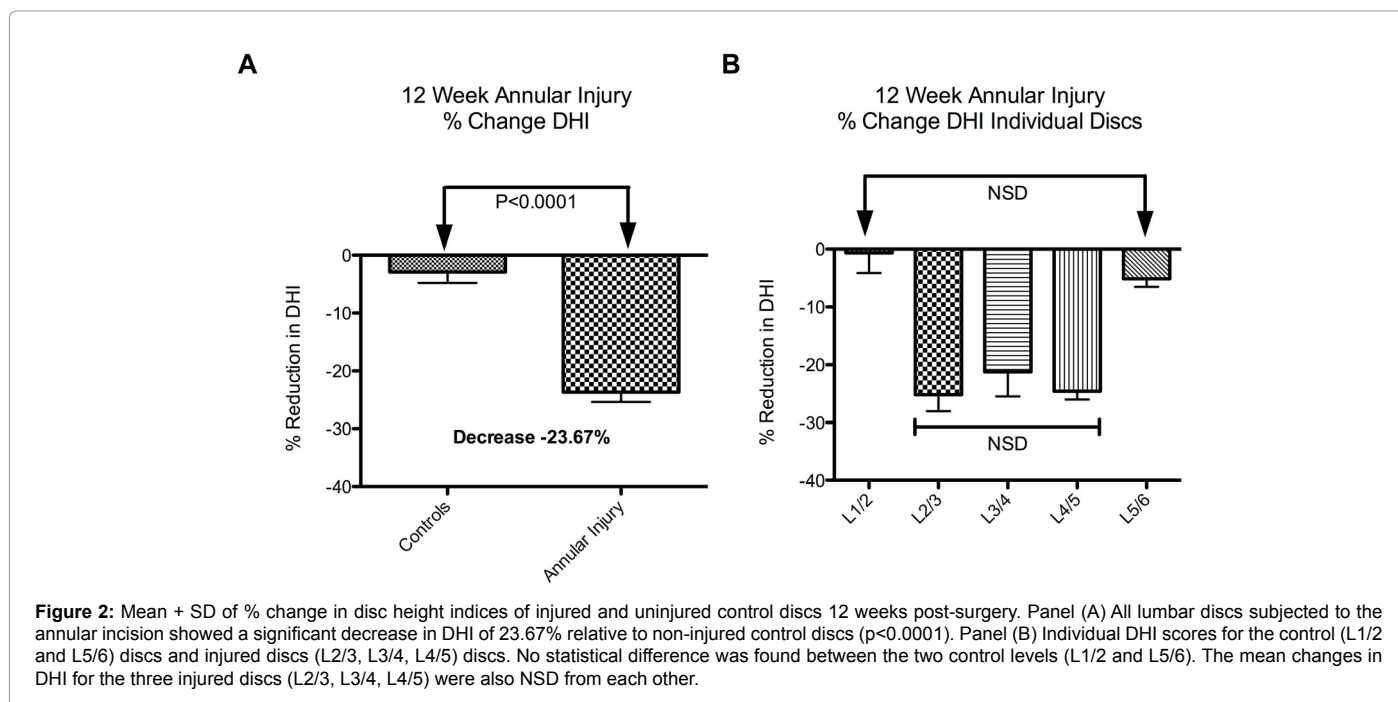
The S-GAG content of the injured AF side (AF1) of the L2/3, L3/4, L4/5 discs were significantly less than the corresponding region of control discs ($p < 0.0005$) (Figure 6C) again signalling a loss of proteoglycans. However, of the contralateral uninjured AF sides of the same lumbar discs failed to show differences to the same region of the control discs (Figure 6D).

No significant differences were observed in collagen content of the NP, AF1 or AF2 of injured or control discs (data not shown).

The total DNA content of the injured AF regions (AF1), as an index of cell numbers, was found to be significantly higher than levels in the non-injured control discs ($p < 0.05$) (Figure 7A). However, no statistical differences were observed between the DNA content of the NP or AF2 and the corresponding control disc regions (Figure 7B, 8C).

Annulus Fibrosis (Score 0 – 10)	
Morphology of AF	0. Well organised 1. Mildly disorganised (<25%) 2. Moderately Disorganised (25-75%) 3. Completely Ruptured (>75%)
Chondrocyte Metaplasia of AF	0. No Chondrocyte Metaplasia 1. Mild chondrocyte proliferation (inner AF layers only) 2. Moderate chondrocyte proliferation (inner half of AF) 3. Marked chondrocyte proliferation (outer AF layers)
Tears and Cleft Formation	0. Absent 1. Rarely present 2. Present in intermediate amounts 3. Abundantly present 4. Scar/tissue defects
Nucleus Pulposus (Score 0 – 9)	
Matrix Staining of NP	0. Red stain dominates 1. Mixture of red and green staining 2. Green stain dominates
Chondrocyte Proliferation of NP	0. No proliferation 1. Increased chondrocyte cell density 2. Connection of two chondrocytes 3. Small clones (2-7 cells) 4. Moderate size clones (8-15 cells) 5. Huge clones (>15 cells) 6. Scar tissue defects
Endplate and Vertebral Body (Score 0 – 9)	
Endplate Morphology	0. Regular homogeneous Structure 1. Slightly irregular thickness 2. Moderately irregular thickness 3. Severely irregular thickness with interruption of endplate
New Bone Formation	0. Absent 1. Minor new bone formation 2. Moderate amount of new bone formation 3. Abundant new bone formation – tendency toward bridging
Subchondral Bone Sclerosis	0. No sclerosis (<2 × thickness of dorsal vertebral cortex) 1. Mild sclerosis (2-4 × thickness of dorsal vertebral cortex) 2. Moderate sclerosis (>4 × thickness of dorsal vertebral cortex) 3. Severe subchondral bone irregularities

Table 2: Histological Scoring criteria used to assess disc histological sections modified from Bergknut's canine histological grading system [28]. The two halves of each disc (e.g. AF1 and AF2) were scored individually according to the above criteria yielding a total disc histological score from 0 (no degeneration) to 54 (extreme degeneration).



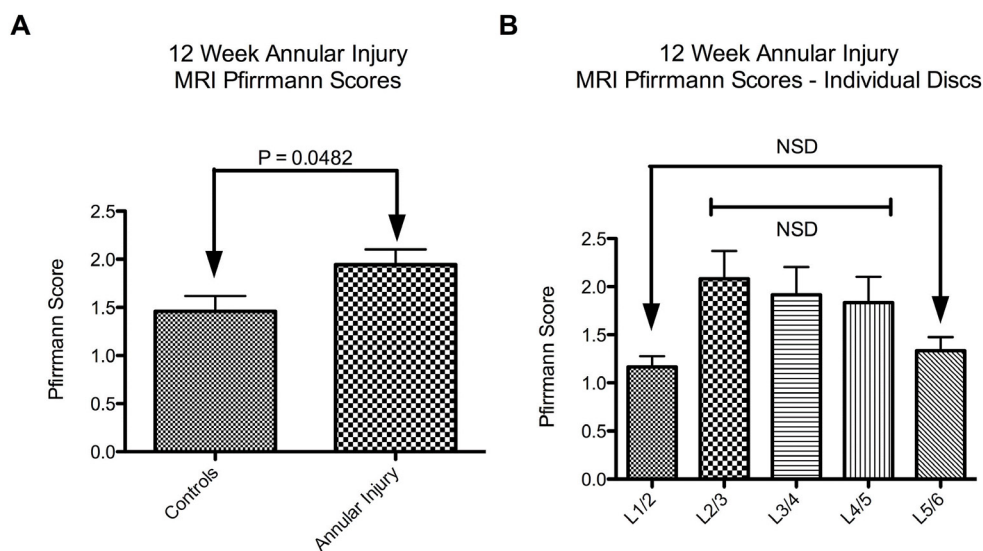


Figure 3: Panel A: Comparison of the mean Pfirrmann MRI scores for the control discs and all injured discs 12 weeks post-surgery. Scores for the operated discs were significantly higher than for the combined uninjured Controls (2.0 versus 1.0, $P=0.0482$). Panel B: Mean Pfirrmann MRI scores for the individual control and injured discs 12 weeks PO. No statistical Differences in scores were observed between the two control discs (L1/2 and L5/6), nor between the three discs subjected to annular incision (L2/3, L3/4, L4/5).



Figure 4: 3T sagittal T2 weighted MRI image of a lumbar spine from animal 9480, sacrificed at 12 weeks. Non-injured adjacent level controls (L1/2, L5/6), represented by blue arrows, and scored Pfirrmann 1. Injured discs L2/3, L3/4 and L4/5, represented by red arrows, scored Pfirrmann 2. As can be seen all injured discs (red arrows) demonstrate an inhomogeneous disc structure with grey banding. Adjacent controls (blue arrows) demonstrate a homogenous bright white structure.

Histological analysis

Examination of the digitised images of the scanned histological sections from discs subjected to annular injury revealed early healing of the outer third of the defect by an amorphous connective tissue that exhibited limited evidence of an organised collagen lamellae structure or the deposition of proteoglycan (Figures 8 and 9). Examination of

the central region of the defect at high magnification showed unhealed lesions with evidence of endogenous fibrocyte/fibrochondrocyte proliferation and tissue repair (Figure 8). In addition, the lesion zone showed the presence of invading capillaries containing erythrocytes and other blood cells that penetrate the collagenous AF matrix (Figure 9F). Noteworthy, was the finding that at the interface of the repaired annular margins the Cartilaginous End Plate (CEP) showed a capillary bud that had penetrated into the repair region and appeared to be releasing its cellular contents into avascular AF region (Figure 9C and 9D). These events contrasted with the same interface of tissues adjacent to normal AF that showed well-defined boundaries between the AF, CEP and vertebral bone plate with no sign of neovascularisation (Figure 9B).

The mean disc histological scores, generated using the modified canine histological grading system [28] were significantly higher in discs undergoing annular injury compared to control levels ($p<0.005$) (Figure 9A). However, on comparing scores for the individual lumbar levels, there were no significant differences between the control levels, or between the three levels that were subjected to annular injury (Figure 9B).

Discussion

The results of the present study have shown, using several independent methods of assessment, that the introduction of a surgical lesion at the periphery of the L2/3, L3/4 and L4/5 lumbar discs of adult sheep induces similar degenerative changes at all three spinal levels. Moreover, the degenerative changes induced were significantly greater than the adjacent uninjured lumbar discs (L1/2 and L5/6).

These findings provide support for the use of this multilevel injury model of disc degeneration to evaluate biological therapies where the active component is, by necessity, administered in a vehicle or preservative such as a cryoprotectant “cocktail” employed to freeze down the cells for storage and transport to the operating room prior to their injection into the disc. The third injured lumbar disc would

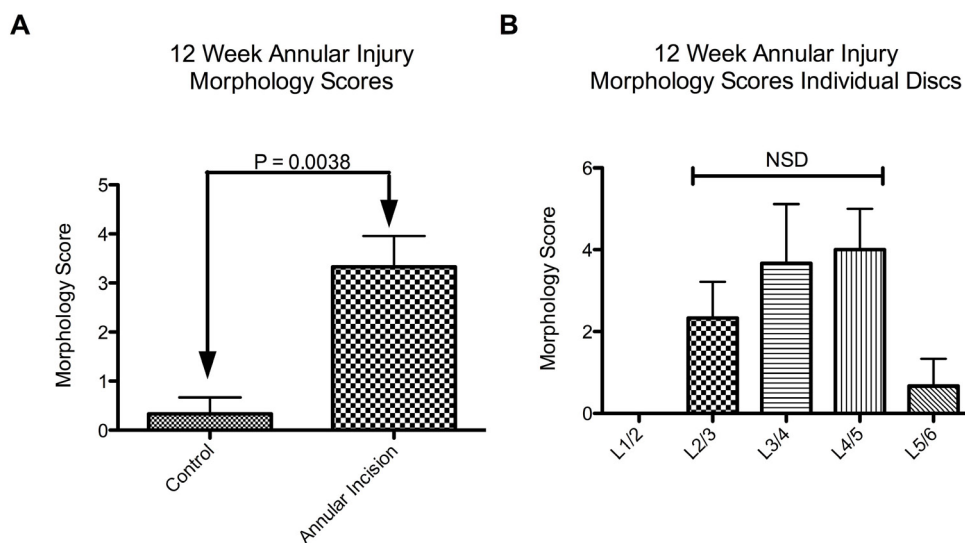


Figure 5: Panel A: Morphology scores were significantly higher in discs undergoing annular injury compared to controls. Panel B: There were no statistical differences between the two control levels, or between the three levels undergoing annular incision.

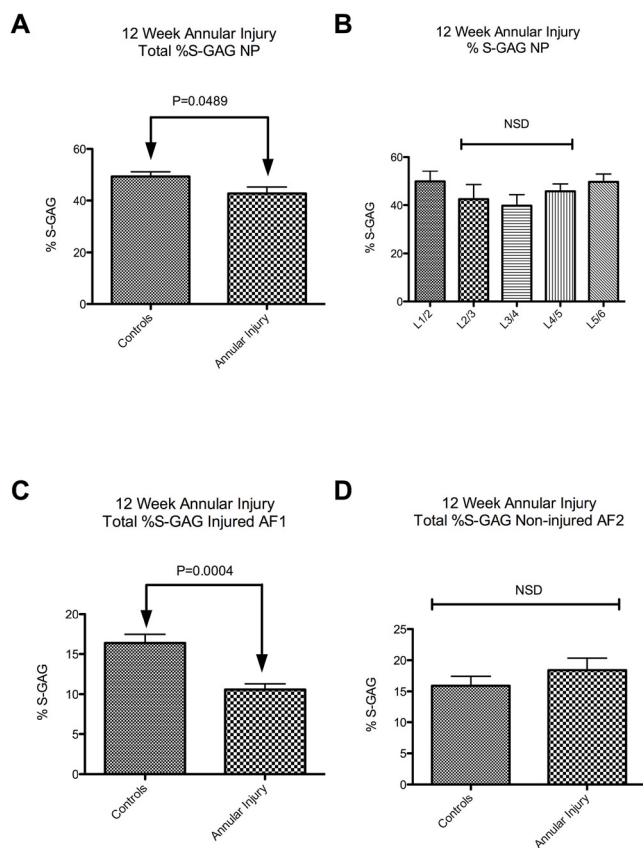


Figure 6: Panel A: Significantly less S-GAG were extracted from the NP of discs undergoing annular injury compared to control discs. Panel B: There were no differences in NP S-GAG content between injured spinal levels. Panel C: Significantly less S-GAGs were extracted from injured AF regions (AF1) compared to controls ($p=0.0004$), whilst there were no differences in the S-GAG content of the contralateral non-injured AF (AF2) (Panel D).

serve as the non-treated negative control. By using the adjacent lumbar discs of the same animal to test the various components present in injectable therapies the number of animals required for the study can be substantially reduced.

The 18 discs subjected to annular injury demonstrated a significant decrease in DHI of 23.67% at 12 weeks when compared to non-injured controls ($p<0.0001$). Loss in disc height is one of the hallmark features of progression in human lumbar disc degeneration. A decrease of 23.67% would be classed as moderate degeneration [43]. Therefore, the changes in disc height observed in the present study would serve as an appropriate starting point to evaluate reparative interventions since a proportion of endogenous disc cell could be expected to be viable and thus receptive to trophic factors release by injected stem cells or other biological regenerative therapies.

An earlier study reported no change in NP T2 signal following annular injury when assessed by 0.27T MRI instrument [41]. In the present study we utilised 3T MRI, which, because of its higher resolution did demonstrate MRI changes in the injured discs. Overall injured discs exhibited Pfirrmann scores of 2.0 compared to the control disc scores that average scores of 1.0. The difference between Pfirrmann 1.0 and 2.0 scores would correspond to the injured discs showing an inhomogeneous structural appearance compared to the bright white homogenous structure found for ‘healthy’ discs (see Figure 4). The histological and morphological grading systems also demonstrated significant differences in degeneration between injured and control discs, a finding that was consistent with the MRI results. As far as we are aware this is the first reported attempt to semi-quantify the histological and morphological changes that occur following annular injury in the sheep model of disc degeneration. Moreover, using this ovine model we have applied the same grading systems to successfully assess histological and morphological changes that occur in ovine degenerative discs following the administration of mesenchymal progenitor cells (MPC) [49,50].

The finding that the proteoglycan content of the NP and AF1 regions discs three months following injury was significantly lower

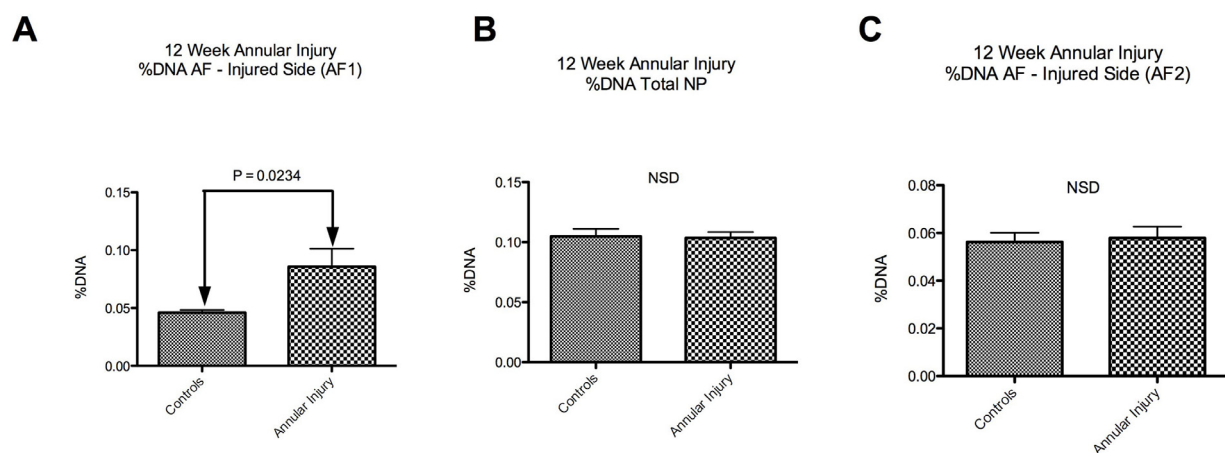


Figure 7: Panel A: The DNA content of the injured AF1 was significantly higher compared to controls. No differences in the DNA content of the NP or non-injured AF were observed (Panels B and C).

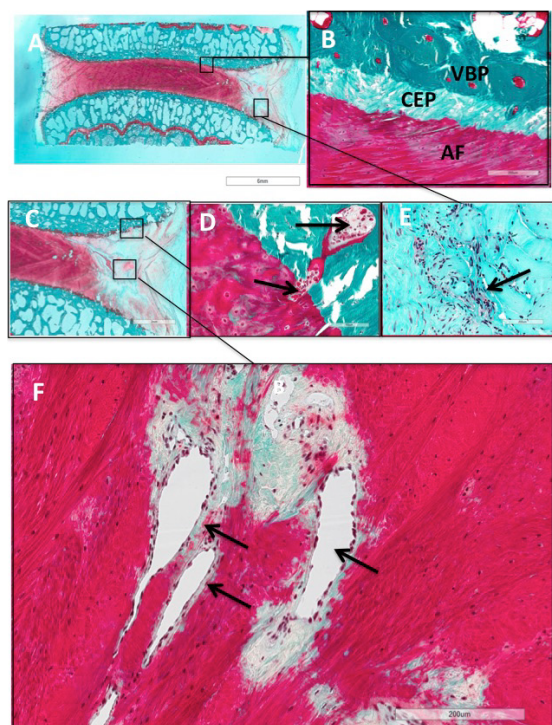


Figure 8: Examples of histological sections from ovine lumbar discs subjected to surgical incision three months previously. Panel A: Coronal methyl methacrylate embedded section stained with Safranin-O/fast-green showing loss of staining for proteoglycans in the AF region subjected to the 6 x 20 mm horizontal incision. Magnification indicated by 6 mm calibration bar. Panel B: shows a the insertion of the fibres of the AF into the cartilaginous end plate (CEP) and the adjacent vascularized vertebral bone plate (VBP) of an unperturbed region of the disc remote to the site of injury. Magnification indicated by 200 µm calibration bar. Panels C: Higher magnification of Panel A showing AF lesion regions adjacent to CEP (boxed) and lesion site (boxed). Panel D shows proliferation and invasion of capillary buds of the CEP into the AF (arrowed). Magnification indicated by 100 µm calibration bar. Panels E and F: shows the presence of capillary vessels at the site of injury (F) and proliferating fibrocytes (arrowed) adjacent to the AF lesion zone. Magnification indicated by 200 µm calibration bar.

than the adjacent non-injured control discs was not unexpected in view of the nature of the annular injury. Previous studies on human degenerate discs have shown that the loss of PGs from these tissues was due to the proteolytic action of Matrix Metallo Proteinases (MMPs) and aggrecanases (ADAMTS 4 and 5) released from disc cells [13,18–21,51,52]. The production of these proteinases by the disc cells of the NP is reported to be due to the action of pro-inflammatory cytokines such as interleukin-1 (IL-1) and possibly tissue necrosis factor-alpha (TNF-alpha) [21,52–54]. The mechanism responsible for the activation of these cytokines and the decreased levels of their endogenous receptor inhibitors is not completely understood but the altered biomechanical status and disruption of disc cell homeostasis are considered to be contributory factors. The situation is compounded by reports that growth factors and their receptors, including Tissue Growth Factor B1 (TGF-B1), Platelet Derived Growth Factor (PDGF), Fibroblast Growth Factor-2 (FGF-2), osteonectin and Vascular Endothelial Growth Factor (VEGF) were up-regulated in disc of this animal model indicating that endogenous repair and neovascularization are also activate in response to annular injury [55,56].

The present biochemical studies also showed that the DNA content of the injured AF region, (AF1), was significantly elevated relative to the contralateral non-injured AF region or AF of control discs. Tissue DNA can be used as a surrogate marker for nucleated cell content, and therefore an elevation of this parameter relative to normal disc tissues could provide an index of endogenous cell proliferation, and vascular invasion. Examination of the histological sections of the injured AF1 region indicated both explanations were valid; however, vascular proliferation and a large influx of blood cells would appear to be the major contributor to the observed elevation in DNA levels.

In conclusion, the present studies have confirmed, using several independent assays, the suitability of this large animal model for evaluating the potential of MSC to reconstitute degenerate ovine lumbar discs relative to their carriers/cryoprotectant in the same animal.

Moreover, since the origin of low back pain experienced by patients with degenerative disc disease has been linked with the ingrowth of nerve fibres and blood vessels from the periphery of the AF to the NP [2,3,24,25] we can now investigate, using this animal model, the potential therapeutic effects of novel modalities of treatment, such as

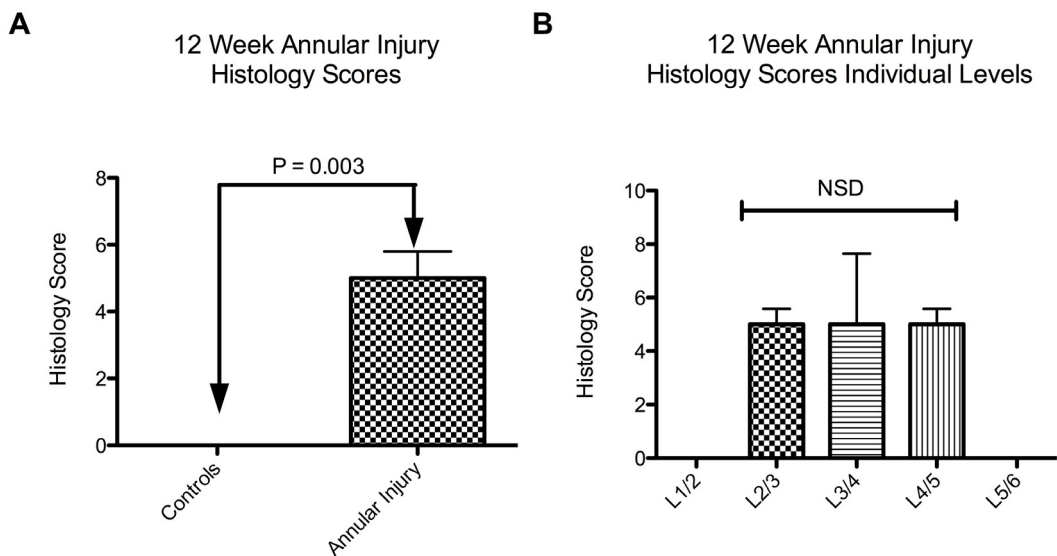


Figure 9: Panel A: Histological degeneration scores were significantly higher in discs undergoing annular injury compared to controls ($p=0.003$). Panel B: There were no statistical differences in histological scores between the two control levels, or between the three lumbar disc levels subjected to annular incisions.

mesenchymal stem cells, to modulate these important pathological pathways.

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